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(19)

(54) METHOD OF ENUMERATING BACTERIA

(71) We, UNIVERSITY OF STRATHCLYDE, incorporated by Royal Charter, of Royal College, 204 George Street, Glasgow G1 1XW, Scotland, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a method of staining micro-organisms. In particular, the present invention relates to a procedure for estimating the extent of microbial contamination in foodstuffs or raw materials used in the production of foodstuffs. However, the field of application of the invention is not limited to food science; it may be used in any situation where the estimation of micro-organisms is required, for example, in the fermentation industries, yeast manufacture, in medical diagnosis and in the manufacture of pharmaceutical products.

The conventional procedure employed in determining the numbers of micro-organisms in a material involves the isolation of the micro-organisms from the material and cultivation of the isolated micro-organisms on a culture medium on a plate. The enumeration of the micro-organisms by this 'colony count' technique is a lengthy procedure which under normal circumstances would take about two days for incubation, depending on the nature and number of the micro-organisms involved, but it is not uncommon for the incubation to take a week or more. When incubation is complete the number of colonies of micro-organisms on the plates is determined visually. This 'colony-count' technique is inherently inaccurate, the errors arising because not all the micro-organisms may be recovered from the material under examination. Some are killed during preparation of the sample before application to the culture medium and also the cultural conditions during incubation may not be appropriate for all the types of micro-organisms in the sample. Thus some of the types may not form colonies and will, therefore, not be estimated by the technique. Additionally, it is known that a single colony may arise from the growth of more than one micro-organism.

By far the most significant disadvantage of the colony-count technique is the length of time required to obtain a result. Often it is the case with a foodstuff that by the time a report has been issued, the material held in storage awaiting the report has deteriorated in quality. It will be appreciated that is it highly uneconomic to store large quantities of food materials, often in expensive refrigerated warehouse space, over lengthy periods of time awaiting a laboratory report on its microbial contamination. It is even more particularly uneconomic if the material is ultimately failed by the test.

Another disadvantage of conventional colony-count methods of enumerating micro-organisms is that only viable cells are counted.

Another technique commonly used in food technology is to stain a glass slide preparation of the foodstuff with a dye such as methylene blue and then to examine the stained smear microscopically. The disadvantage of this method is that the food itself becomes stained, forming a coloured background against which the micro-organisms are difficult to detect. The method has some value as a coarse measurement of contamination in samples of high levels of microbial contamination. At low levels of contamination, the test is inaccurate.

In diagnostic medicine a qualitative technique known as the "fluorescent-antibody technique" (F.A.T.) is used. In this technique, a fluorochrome dye is chemically combined with the antiserum of a specific group of micro-organisms. The fluorochrome-labelled antiserum is mixed with an

extract from the material under test and, if the specific group of micro-organisms is present, a reaction takes place which results in making the specific group of micro-organisms fluoresce on illumination. While this technique is useful for the identification of specific groups of micro-organisms, it is too specific for quantifying the general microbial content of a material.

An object of the present invention is to provide a method of staining micro-organisms with a fluorochrome dye to obtain enhanced fluorescence under U.V. illumination.

According to the present invention there is provided a method of staining micro-organisms comprising providing a suspension of micro-organisms in a liquid medium, reacting the micro-organisms in the suspended state with phosphate ions whereby phosphate ions react at active sites on the micro-organisms to produce phosphate derivatives of the micro-organisms having at said sites polydentate phosphate groups, and treating said phosphate derivatives with a phosphate-reactive fluorochrome dye which thereby combines chemically with the micro-organisms via the intermediate phosphate groups.

Preferably the said phosphate derivatives are treated in the suspended state in liquid medium with said fluorochrome dye to produce a liquid specimen for microscopic examination in the liquid state.

Alternatively said phosphate derivatives are applied to a carrier substrate and fixed thereto using a liquid fixative and the fixed sample is brought into contact with the fluorochrome dye to be stained, the stained sample being dried to provide on the substrate a dry specimen for microscopic examination in the dry state.

The staining may be enhanced by additional treatment of the micro-organisms by any one or more of the following chemical treatments selected from methylation, esterification, hydrolysis, oxidation, and treatment with sulphur dioxide.

Methylation may be effected by treating the sample on a carrier plate with an ethereal solution of diazomethane. Esterification may be effected with a sulphuric acid ester, preferably with control of time, temperature and pH.

Hydrolysis may be effected by treatment of the sample on a carrier plate with an acid selected from hydrochloric, perchloric, periodic, sulphuric or nitric. Oxidation may be effected with an oxidising agent. Preferably, the time, temperature and pH of the treatment are controlled.

The treatment with sulphur dioxide may simply involve exposure of the sample on the carrier plate to a solution of sulphur dioxide or a salt thereof capable of releasing sulphur dioxide or a solution of thionyl chloride. Time, temperature and pH of the treatment are preferably controlled.

The method of the invention may be used to produce specimens or alternatively dry, fixed specimens on a carrier plate. The wet technique has the advantage of rapidity whereas the advantage of the dry technique is that the sample may be subjected to a multiplicity of treatment steps without large volumes of reagent solutions accumulating. In the fixed sample preparations, between each treatment step the sample on the plate may be washed in water or a buffer solution preferably under controlled conditions of time, temperature and pH.

The dry preparation may be produced by applying a liquid sample of the phosphate derivatives to a carrier plate such as a microscope slide, a plastics film or an opaque plate or strip. For enumeration of the micro-organisms, a known volume of sample is applied to a known area of the carrier plate. After application of the sample to the plate the liquid is allowed to evaporate at ambient or elevated temperature. In practice, a unit volume of a liquid sample is applied to a carrier plate and the liquid allowed to evaporate either naturally or by application of heat. The cells of the micro-organisms may then be fixed on the slide by heating same or by immersion in a solvent such as alcohol, acetone, acetone-alcohol solution, alcohol-acetic acid solution and formaldehyde, followed by drying of the fixed preparation.

The fluorochrome dye may be selected from the group consisting of lissamine-rhodamine B, acridine orange, acridine yellow, primuline, ethidium bromide, acriflavine, eosin Y, auramine, tetramethyl-oxamethine-cyanine ester, rhodamine B, rhodamine 3G, fluorescein, fluorescein diacetate and thionin. The staining may be carried out simply by adding a solution of fluorochrome dye or dyes to a suspension of the phosphate derivatives or by immersing the plate carrying a fixed sample of the derivative in a solution or solutions of a dye or dyes, removing the plate, washing off excess dye or dyes and drying the plate in air or by the application of heat. The staining may be carried out under controlled time, temperature, pH and light conditions.

It is possible to use, in the performance of this invention, traditional staining techniques such as counter-staining to mask background interference. Also, it is sometimes advantageous to incorporate a treatment with an optical brightener to intensify fluorescence of the dye.

The stained preparation is examined under a microscope to enumerate or study

the micro-organisms therein.

While not wishing to be bound by any particular theory it is believed that the staining technique of this invention involves, at a basic level, reaction of the dye with D.N.A. molecules in the cells of the micro-organisms. When a micro-organism is exposed to a dye, one molecule of dye links to the micro-organism at each of certain locations in the D.N.A. molecule (dye-receptor sites). Treatment with phosphate ion forms at each dye-receptor site a phosphate bridge to which more than one molecule of dye may then link. Thus the uptake of dye by the micro-organism is increased, resulting in increased fluorescence from each micro-organism when viewed under U.V. light.

Each of the additional treatments mentioned above, i.e. methylation, esterification, hydrolysis, oxidation and treatment with sulphur dioxide, it is believed, modify the D.N.A. molecule to form additional dye-receptor sites. It is thought that the formation of additional aldehyde and carboxylic acid groups contributes to the reaction mechanism.

The invention also permits discrimination between viable and non-viable micro-organisms and it may be tentatively explained as follows: When a micro-organism is viable the cell membrane acts to retard penetration of the dye into the cell thus restricting contact between the dye and the D.N.A. resulting in diminished uptake of dye. When the micro-organism is non-viable the cell membrane is in a ruptured condition and therefore presents no barrier to penetration by the dye resulting in enhanced uptake of dye. The effect is that because of the differential absorption the stained viable and non-viable micro-organisms fluoresce at different wavelengths: for example, using acridine orange dye, the viable micro-organisms fluoresce green of wavelength 540 nm and non-viable micro-organisms fluoresce orange-red of wavelength 665 nm.

When a dry preparation of micro-organisms is used it is possibly steric effects in the D.N.A. molecule which enable viable/non-viable discrimination. The effect of fixing the phosphate derivatives of the micro-organisms to a slide kills them but in those which were viable prior to treatment with phosphate and fixing the D.N.A. molecules retain the highly ordered double-helix structure whereas in those which were non-viable the helices are disorganised. Disorganisation of the D.N.A. structure results in a reduced number of available dye-receptor sites due, probably, to steric hindrance to absorption of the phosphate and dye. Thus viable micro-organisms are characterised by a high degree of absorption with associated high fluorescence (e.g. yellow-orange, 630 nm and acriflavine) and non-viable by low fluorescence (e.g. green, 540 nm with acriflavine).

An advantage of this invention is that it makes possible the counting of not only viable micro-organisms (as with the standard colony-count technique) but also non-viable micro-organisms. The non-viable cell count is of importance in that it indicates the microbiological history of the sample. For example, if a manufacturer were to sterilise a spoiled foodstuff then a standard colony-count would show the absence of viable micro-organisms. However, the present method would reveal that at some time the food had contained numbers of micro-organisms.

To enumerate the micro-organisms in the sample the area of stained preparation may be visually scanned under the microscope from the top to the bottom and from side to side. Alternatively the micro-organisms may be enumerated by means of an image analysing system using a photomultiplier sensing device which provides a digital readout of units, such as micro-organisms fluorescing at a given wavelength.

The cells may be counted visually using a scanning technique as follows:—

Depending on the number of bacteria present in the sample the number of fields examined was generally between 16 and 40. The reasons for the selection of this range of fields will be explained in the discussion. The movement between fields was performed blind to ensure a random selection of fields.

The mean number of organisms per field may be calculated knowing the total number of bacteria counted and the number of fields viewed. This figure is multiplied by a factor of 982 to give the total number of bacteria in 10μl. The degree of precision of each calculation was determined by reference to tables of Schedules of Precision, Cassel.

Examples of results are reported in the Tables below which give, for comparison, the results obtained by a colony-count on the same sample:

Tables 1 to 4 show the results of colony-counts and microscope counts (orange-fluorescing cells) of meat samples inoculated with different test strains. Column A shows the mean colony-count per gram and column B the standard deviation of this value. Column C shows the calculated number of viable micro-organisms by the technique of this invention and column D gives the 90% Confidence Interval of this value. Column E shows number of micro-organisms counted and the number of fields viewed.

Replicates of plate counts obey a normal

distribution. The distribution in the case of microscope technique is Poisson in nature but can be approximated by the normal distribution when more than 15 organisms are counted. The Confidence Interval (Column D) predicts with 90% probability the limits of the "true" values of micro-organisms per gram from the Observed value (Column C).

It can be seen that the higher the number of micro-organisms counted the smaller the value of the 90% Confidence Interval and the greater the precision of the technique.

the reproducibility of the counting of stained preparations prepared according to the method described above is shown in Table I Expt. 1. Table 2 Expts. 1, 2, 3, Table 3 Expts. 1, 4 and Table 4 Expts. 1 and 2 where two or more preparations were counted for each sample.

TABLE 1

	Colony count		Enumeration by the Method of invention		
E. coli	A mean/gram	B S.D.	C number/gram	90% Confidence D interval	Number of bacteria E and fields counted
Expt. 1	11.4×10^6	± 0.7	11.2×10^6	± 1.34 (± 12%)	190 : 17
			10.0×10^6	± 1.20 (± 12%)	158 : 16
2	7.8×10^6	± 0.9	9.0×10^6	± 1.07 (± 12%)	179 : 20
3	5.25×10^6	± 0.4	7.2×10^6	± 1.08 (± 15%)	123 : 25
4	2.08×10^6	± 0.14	2.9×10^6	± 0.53 (± 18%)	73 : 25
5	1.31×10^6	± 0.2	3.1×10^6	± 0.75 (± 24%)	52 : 17
6	1.15×10^6	± 0.13	1.3×10^6	± 0.51 (± 39%)	24 : 19
Meat Control	1.0×10^2 – 6.6×10^2		less than 4×10^3		0 : 30

TABLE 2

Ps. Fluorescens	Colony count A mean/gram	B S.D.	Enumeration by the Method of invention C Number/gram	D 90% Confidence interval	E Number of bacteria and fields counted
Expt. 1	1.92×10^6	± 0.28	2.17×10^6	± 0.36 (± 15%)	107 : 40
			1.60×10^6	± 0.32 (± 20%)	65 : 32
2	1.17×10^6	± 0.30	8.6×10^5	± 2.3 (± 27%)	38 : 48
			9.7×10^5	± 3.1 (± 32%)	24 : 27
3	3.2×10^5	± 0.18	4.5×10^5	± 1.24 (± 27%)	31 : 39
			4.25×10^5	± 1.51 (± 31%)	26 : 34
Meat Control	1.5×10^3		less than 4×10^3		

TABLE 3

Staph. aureus	Colony count A mean/gram	B S.D.	Enumeration by the Method of invention C number/gram	D 90% Confidence interval	E Number of bacteria and fields counted
Expt. 1	1.32×10^6	± 0.27	1.95×10^6	± 0.30 (± 18%)	78 : 24
			1.92×10^6	± 0.38 (± 22%)	54 : 17
			1.66×10^6	± 0.39 (± 28%)	37 : 30
2	4.28×10^5	± 0.65	5.85×10^5	± 2.28 (± 45%)	15 : 29
3	1.62×10^5	± 0.39	1.32×10^5	— (50%)	8 : 36
4	3.47×10^4	± 0.73	2.64×10^4	— (50%)	4 : 23
			2.00×10^4	— (50%)	3 : 23
Meat Control	6.0×10^2		less than 4×10^3		0 : 30

TABLE 4

Bacillus sp.	Colony count A mean/gram	B S.D.	Enumeration by the Method of invention C number/gram	D 90% Confidence interval	E Number of bacteria and fields counted
Expt. 1	5.9×10^4	± 1.8	1.58×10^5	– (50%)	6 : 38
			1.97×10^5	– (50%)	6 : 28
2	4.2×10^4	± 0.6	5.4×10^4	– (50%)	2 : 36
			3.96×10^4	– (50%)	2 : 45
Meat Control	2×10^3		less than 4×10^3		0 : 30

The colony-counted method appears to give a smaller variation, in terms of standard deviation, then microscopic enumeration. However, the standard deviation and 90% Confidence Interval are not comparable. It can be said that the accuracy of the plate count was artificially enhanced by the use of 5 replicates, a situation which does not occur in routine quality-control procedures.

Many samples displayed close correlation betwen mean colony-counts and microscopic enumeration by the technique of the invention, the latter always being within the corresponding values of the colony-counts ± standard deviation.

In general enumeration using the microscopic technique was higher than the corresponding colony-count. This could be due to:

a) the microscopic method using slides stained by this invention will estimate micro-organisms singly whereas colonies may derive from a single micro-organism or a group of bacteria.

b) there is the likelihood that a certain proportion of viable cells will not grow in laboratory recovery media, but which may multiply in foodstuffs. This is particularly relevant to "stressed" bacteria and bacterial spores. The units are still viable and are counted.

Microscope counts of meat or vegetable washings do not pose great problems regarding background fluorescence and the physical masking of fluorescing micro-organisms. However, other foods such as milk or egg powder gave such high background fluorescence that counts were difficult using the standard technique. Short

fixation times in alcohol with subsequent treatment in 2% acetic acid successfully removed such background fluorescence. Unfortunately, this process also caused many of the micro-organisms to be removed from the slide. In further experiments immersion of the preparation in a Coplin jar of water for 1 minute after treatment with alcohol-acetic acid for 30 minutes indicated that with modifications the microscopic technique was applicable to milk and egg based foods.

Using the staining technique of the invention it was found that old cultures, particularly Gram negative rods, showed bright orange fluorescence together with dull green fluorescent cells. Cultures of Gam negative bacteria sterilised by boiling at 100°C for 30 minutes fluoresced uniformly dull green. This change from orange to green fluorescence was shown to be related to viability of the test culture. While old cultures of *Staphylococcus aureus* displayed both orange and green fluorescent cells the heat treatment of a young culture by boiling for 30 minutes with subsequent staining showed all organisms to be orange fluorescent. However, pre-treatment of the preparation with buffer solutions obviated such 'false-viable' reactions. The differences in reactions between Gram negative and Gram positive bacteria towards the fluorochromes illustrate important compositional differences between the two groups.

An advantage of the invention is the rapidity with which results can be obtained. The staining method of the invention takes less than one hour for the actual staining procedure while the conventional colony-count requires several days incubation. Also the possibility exists of using a rapid optical scanning instrument to enumerate or detect the fluorescent cells. By adjusting the wavelength sensitivity of such an instrument it will be possible to determine separately viable and non-viable cells.

The invention will now be described by the following Examples which describe only the staining steps and the results obtained without discussing the counting technique.

Example 1
Wet preparation: Acridine orange stain

Procedure

1. A milk sample containing micro-organisms is pre-mixed with 0.62M phosphate buffer pH 7.2 to a given dilution.
2. One ml. of sample is pipetted into a container to which is added 1 ml of 0.01% solution of acridine orange in 0.62M phosphate buffer pH 7.2.
3. After agitation for 2 minutes at 25°C 0.02 ml of the sample mixture is pipetted on to the cleaned and dried carrier plate.
4. The applied sample is then covered with a clean, dry cover glass.
5. The micro-organisms in the preparation are then counted by incident illumination through a X40 objective using a 450 n.m. excitation filter, a Ploem 3 dichroic mirror and a 530 n.m. barrier filter.

Comments

This staining procedure, which is also applicable to food and urine samples, results in viable micro-organisms being predominantly green (540 nm) while the non-viable micro-organisms predominantly stained orange-red (665 nm).

The adjustment of the pH of the acridine orange solution to 7.2 from the normal pH of 4.5 enhanced the uptake of the fluorochrome by the nuclear material of the micro-organisms and the use of phosphate ions in the diluting buffer assisted the uptake of the fluorochrome and improved the level of emission of the fluorescing micro-organism.

This technique is suitable for materials containing low levels of contaminating proteins, fats and other background material.

The mechanism of staining by acridine orange in this wet preparation is primarily due to binding with the nucleic acid. In viable micro-organisms the cell membrane regulates the intake of fluorochrome and the highly ordered state of the nucleic acids and proteins allows for minimal binding of the fluorochrome resulting in emission of green fluorescence at around 540 nm. The non-viable micro-organisms lose the selective permeability of the cell membrane, resulting in the provision of acidic and aldehydic groups to which the fluorochrome will bind in great quantities, giving orange-red fluorescence at 665 nm.

Example 2
Wet preparation: Total Count in Urine

Procedure

1. Urine samples are diluted with 1:1 volumes of 0.5M phosphoric acid and 0.2M oxalic acid pH 2.0 to effect acid hydrolysis.
2. Add 0.2 ml of 0.01% ethidium bromide and hold at 40°C for 10 minutes.
3. The sample is adjusted to pH 6.5 using 0.5M disodium hydrogen phosphate in solution.
4. Proceed as in Example 1 steps 3 to 5 at pH 7.2.

Comments

This procedure was designed to disorganise the nucleic acid and tertiary structure of proteins in the microbial cell by

acid hydrolysis resulting in the formation of acidic and aldehydic groups. These groups in the presence of phosphate groups at pH 6.5 bind large numbers of ethidium bromide molecules resulting in all micro-organisms showing orange-red fluorescence.

Example 3

Wet Preparation: Pre-irradiation of Yeast Stain

Procedure

1. One ml. of a sample containing yeasts in aqueous suspension is combined with 1 ml of a saturated aqueous solution of fluorescein diacetate in 0.62M phosphate buffer at pH 6.8 and held at 25° for 30 seconds.
2. To the above suspension is added 2 ml. of 0.01% acridine orange in M/15 phosphate buffer at pH 7.2.
3. Proceed as in Example 1 steps 3 and 4.
4. Irradiate the prepared sample at 450 nm for 5 seconds.
5. Illuminate the prepared sample at 360 nm and count yeast cells using the Ploem 1 dichroic mirror and a 430 nm barrier filter.

Comments

The reason for the enhancement of the levels of emission of yeast cells by pre-irradiation at 450 nm is not clear. It is considered that the preliminary treatment of yeast cells with fluorescein diacetate provides an initial link with active sites; the effect of acridine orange in the presence of phosphate ions being to link with the fluorescein diacetate. Pre-irradiation at 450 nm may photo-catalyse a reaction between the fluorochrome groups, reducing the quantity of excitational energy converted into thermal energy, producing a high quantum yield of fluoroscence at the longer wavelength.

Example 4

Wet preparation: Total staining of bacteria in milk or urine samples

Procedure

1. 1 ml of milk is mixed with 1 ml of 0.025% aqueous acridine yellow in 0.62M phosphate buffer at pH 5.6 and held for 30 seconds at 25°C.
2. Two ml. of a mixture of 2% orthophosphoric acid and 8% tartaric acid is added to the preparation to give a pH of 1.5 and the preparation held at 40°C for 10 minutes.
3. Adjust to pH 6.5 using 0.5M sodium dihydrogen phosphate solution.
4. 0.02 ml of the sample is pipetted on to a cleaned and dried carrier plate.
5. Proceed as for Example 1, steps 4 and 5.

Comments

All bacteria fluoresced a uniform bright yellow (575 nm) against a dark background with no staining of the fat globules or milk-protein.

Acridine yellow has a similar affinity for microbial DNA as acridine orange but gives much less non-specific background fluorescence in this wet preparation.

Example 5

Wet preparation: Total bacteria in milk or urine sample

Procedure

1. One ml of milk sample is diluted to 1:10 with water.
2. Prepare a 1.0% solution of Sudan Black B in Cellosolve (Trade Mark) and dilute to 0.1% in water containing 0.25% sodium hexametaphosphate pH 6.5. With agitation add 1 ml of this system to the milk sample and hold for 1 minute at 25°.
3. Add, with agitation, 1 ml 0.15% tetra methyl-oxamethine-cyanine ester in water and hold for 1 minute at 25°.
4. 0.02 ml of the sample is then pipetted on to a cleaned and dried carrier plate.
5. Proceed as in Example 1 steps 4 and 5 using excitation at 360 nm, the Ploem 1 dichroic mirror and the 430 nm barrier filter.

Comments

Treatment of the milk sample with Sudan Black B eliminates background staining by fat globules and the agitation during addition of the reagents ensures the submicroscopic precipitation of the casein fraction. Bacteria stain an intense blue colour (480 nm) against a black background.

Example 6

Wet preparation: Total count in milk samples

Procedure

1. To 1 ml of a 1:10 dilution of milk in water add 1 ml of 1% aqueous solution of chloroxylenol pH 8.5. Allow to act for 30 seconds at 25° with agitation. Chloroxylenol is a defatting agent.
2. Add 1 ml of 0.5% malachite green (a background stain) in water pH 3.5 and allow to act for 30 seconds at 35° with agitation.
3. Add 1 ml of 0.01% acridine orange in M/15 phosphate buffer pH 7.2. Allow to act for 2 minutes at 25°.
4. 0.02 ml of the sample is then pipetted on to a cleaned and dried carrier plate.
5. Proceed as in Example 1 steps 4 and 5 employing excitation at 450 nm, the

Ploem 3 dichroic mirror and the 530 nm barrier filter.

Comments

In this procedure the ability of malachite green to react with milk fat and casein was utilised to block access to the fluorochrome.

Using a pretreatment with chloroxylenol the permeability of the bacteria towards acridine orange was enhanced.

Using this system bacteria fluoresced a bright green-yellow 565 nm against a blue-green background 520 nm. There was little evidence of fat globules or casein in these preparations.

Example 7

Wet preparation: Total count in urine (high contrast stain)

Procedure

1. 1 ml urine is mixed with approximately 1 ml 0.75M orthophosphoric acid to pH 2.0.
2. Add 0.5 ml, 0.1% aqueous sodium thiosulphate and 0.2 ml, 0.01% aqueous ethidium bromide and hold the mixture at 40° for ten minutes.
3. Add 0.5 ml, 0.05% aqueous sodium hypochlorite and adjust the pH to 6.5 using 0.5M disodium hydrogen phosphate solution.
4. Prepare and examine as in Example 6, steps 4 and 5.

Example 8

Dry preparation: Acriflavine Stain

Procedure

1. A milk sample containing micro-organisms is pre-mixed with 0.62 phosphate buffer pH 7.2 to a known dilution.
2. A 0.01 ml aliquot of the sample is applied to a clean dry carrier plate and spread on 1 sq. cm. using a sterile applicator.
3. The applied sample is dried at 25°C.
4. The applied sample is fixed to the carrier plate by covering with 95% (w/v) ethanol for 10 minutes at 25°C.
   Alternative fixative solutions also used include a) 1:1 vols. 95% ethanol:20% acetic acid b) 10% formalin.
5. The fixed preparation is now drained and rinsed with 0.62 phosphate buffer to pH 7.2.
6. The preparation is then covered with 0.01% acriflavine in 0.62 phosphate buffer at pH 7.2 for 10 minutes at 25°C.
7. The preparation is then washed with water and allowed to dry in air at 25°C.
8. The preparation is then counted by incident illumination, without a cover glass, through a X40 objective using an excitation of 450 nm using a Ploem 3 dichroic mirror and a 530 nm barrier filter.

Comments

This staining procedure which is also applicable to food and urine samples, results in viable micro-organisms being predominantly stained yellow-orange while non-viable micro-organisms predominantly stain green.

Under the conditions of staining of micro-organisms by this dry preparation it is considered that the binding of the fluorochrome acriflavine occurs at the cell wall with layers of fluorochrome being built up. In viable cells this results in a high concentration of acriflavine units resulting in the yellow-orange emission wavelength of 630 nm. In non-viable cells the disorganisation of the binding sites on the cell wall results in the absorption of acriflavine only at low levels, thus providing a green fluorescence at 540 nm.

Example 9

Dry preparation: Viable-Non-viable differentiation of Gram negative bacteria

Procedure

1—5 Sample prepared as in Example 8 of steps 1 to 5.

6. The preparation is then immersed in a bath containing 1N hydrochloric acid at 60°C for 5 minutes to effect acid hydrolysis.
7. The preparation is then removed and washed in water.
8. The preparation is then immersed in a solution containing 1 ml of 5% acriflavine and 10% potassium metabisulphite in 0.1N hydrochloric acid diluted with 5% potassium metabisulphite in 0.1N hydrochloric acid for 10 minutes at 25°C.
9. The preparation is then removed, washed with water and dried in air at 25°C.
10. The preparation is then examined as in Example 8, step 8.

Comments

This staining procedure results in the better differentiation of viable (orange-fluorescent) from non-viable (green-fluorescent) Gram negative micro-organisms.

The acid hydrolysis treatment (step 6) releases available aldehydic groups in the D.N.A. molecule, forming, in effect a polyaldehyde. In the viable micro-organism the D.N.A. is highly ordered and the resultant polyaldehyde is also highly ordered. The sulphur dioxide applied in step 8 combines with the aldehyde groups and allows the fluorochrome to form an equally spaced

matrix and the associated complex results in a massive uptake of acriflavine with resultant yellow-orange fluorescence.

In the non-viable Gram negative micro-organism the D.N.A. is denatured and the resultant polyaldehyde highly unordered resulting in poor association of the acriflavine, low uptake and therefore the emission of green fluorescence. By the application of heat to the sample for 10 minutes at 115° prior to step 6, differentiation between Gram positive and Gram negative bacteria may be obtained. Following this procedure Gram positive bacteria fluoresce orange and Gram negative bacteria fluoresce green.

Example 10

Dry preparation: Viable-Non-viable differentiation of Gram positive bacteria

Procedure

1. Samples containing cultures of Gram positive micro-organisms e.g. *Staphylococcus aureus* were diluted 1:1 with 0.1M sodium barbitone-hydrochloric acid buffer at pH 5 containing 100 p.p.m. calcium. Each suspension was heated for 10 minutes at 121°C and then cooled rapidly to 4°C.
2. Proceed as for Example 8, steps 2 to 5.
3. Proceed as for Example 9, steps 6 to 10.

Comments

The staining of Gram positive bacteria by acriflavine differs from that shown by Gram negative bacteria. *Staph. aureus* differs from Gram negative bacteria in that it contains teichoic acid, a ribitol phosphate polymer, in the cell wall and also contains greater quantities of polysaccharides than do Gram-negative bacteria.

In *Staph. aureus* it is considered that acriflavine combines strongly with teichoic acid, through phosphate ester links, and/or with the polysaccharide fraction also through phosphate or ester links.

The replacement of phosphate buffer by barbitone buffer reduces the stability of these ester links and the calcium included in the heating system blocks other phosphate groups.

Thus, following step 1 *Staph. aureus* demonstrates a non-viable i.e. green fluorescent staining reaction compared with a viable staining reaction showed in its absence.

Example 11

Dry preparation: Gram positive bacteria in milk

Procedure

1. A 1.10 dilution of milk in water is prepared and fixed with alcohol as described in Example 8 steps 1 to 5.
2. The fixed preparation is immersed in 1.0% aqueous 4,4'-diamino 2,2'-stilbene-disulphonic acid ester for 5 minutes at 25°C.
3. The preparation is then rinsed in water at 25°C.
4. The preparation is then immersed in 0.01% acridine orange in 0.62 phosphate buffer at pH 7.2 for 5 minutes at 25°C.
5. The preparation is then examined as in Example 1 step 5.

Comments

Using this procedure Gram positive bacteria were found to show orange fluorescence while Gram negative bacteria were a dull brown colour. The general background was green/blue with low interference by casein and the milk fat globule membrane. The uptake of acridine orange by casein and the fat globules was blocked by step 2.

WHAT WE CLAIM IS:—

1. A method of staining micro-organisms comprising providing a suspension of microorganisms in a liquid medium, reacting the micro-organisms in the suspended state with phosphate ions whereby phosphate ions react at active sites on the micro-organisms to produce phosphate derivatives of the micro-organisms having at said sites poly-dentate phosphate groups, and treating said phosphate derivatives with a phosphate-reactive fluorochrome dye which thereby combines chemically with the micro-organisms via the intermediate phosphate groups.

2. A method according to claim 1, in which the said phosphate derivatives are treated in the suspended state in liquid medium with said fluorochrome dye to produce a liquid specimen for microscopic examination in the liquid state.

3. A method according to claim 1, in which said phosphate derivatives are applied to a carrier substrate and fixed thereto using a liquid fixative and the fixed sample is brought into contact with the fluorochrome dye to be stained, the stained sample being dried to provide on the substrate a dry specimen for microscopic examination in the dry state.

4. A method according to any of claims 1 to 3, in which the micro-organisms are subjected to chemical modification to increase the number of active sites thereon, said modification being one or more chemical treatments selected from the group consisting of methylation, esterification, hydrolysis, oxidation and reaction with sulphur dioxide.

5. A method according to any preceding claim, in which the fluorochrome dye is

selected from the group consisting of lissamine-rhodamine B, acridine orange, primuline, ethidium bromide, acriflavine, tetramethyl-oxamethine-cyanine ester, eosin Y, auramine, rhodamine B, rhodamine 3G, fluorescein, fluorescein diacetate and thionin.

6. A method according to claim 5, in which the phosphate derivatives are additionally treated with an optical-brightening agent.

7. A method according to claim 6, in which the optical brightening agent is 4,4'-diamino-2,2'-stilbenedisulphonic acid ester.

8. A method of enumerating micro-organisms comprising preparing a suspension of micro-organisms in a liquid medium, adding to the suspension a solution of phosphate ions thereby to produce a suspension of phosphate derivatives of the micro-organisms, treating the suspended phosphate derivatives with a solution of fluorochrome dye to stain said derivatives, and counting the number of stained micro-organisms in said stained suspension in a thin layer under a microscope with illumination of the stained suspension with fluorescence-activating ultra-violet light.

9. A method according to claim 8, in which said phosphate ions and said fluorochrome dye are added to the said suspension in a single solution containing both.

10. A method of enumerating micro-organisms comprising preparing a suspension of micro-organisms in a liquid medium, adding to the suspension a solution of phosphate ions thereby to produce a suspension of phosphate derivatives of the micro-organisms, applying a measured sample of the said derivatives to a carrier substrate, drying the sample, fixing the dry sample to the substrate by means of a liquid fixative, immersing the fixed sample in a solution of a fluorochrome dye to stain same, drying the stained sample and counting the number of stained micro-organisms by examination under a microscope with illumination of the sample with fluorescence-activating ultra-violet light.

11. A method of staining micro-organisms substantially as hereinbefore described with reference to any one of the Examples.

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